

Enhanced Vascularization of a Human Skin Substitute Engineered with a Non-Viral Vector to Secrete Elevated Levels of Angiogenic Factors

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Abstract

Purpose

Formation of highly-vascularized granulation tissue is essential to expedite the healing of acute and chronic wounds. Current skin substitute tissues rely on expression of endogenous factors and are not designed to promote angiogenesis and granulation. The goal of this study is to develop second generation skin substitutes specifically designed to promote robust vascularization and healing in these types of wounds by expressing elevated levels of angiogenic factors.

Methods

The well-characterized, long-lived NIKS[®] keratinocyte cell line was stably transfected with non-viral plasmid vectors encoding either a single angiogenic factor (VEGF) or a transcriptional regulator that induces multiple angiogenic factors in response to hypoxia (HIF-1 α). Skin substitute tissue prepared from the modified cells (ExpressGraft^{vascular} tissue) was evaluated for expression of the transgenes and secretion of angiogenic factors. Vascularization of ExpressGraft^{vascular} tissue was quantified by CD31 staining nine days after engraftment on diabetic and nude mice.

Results

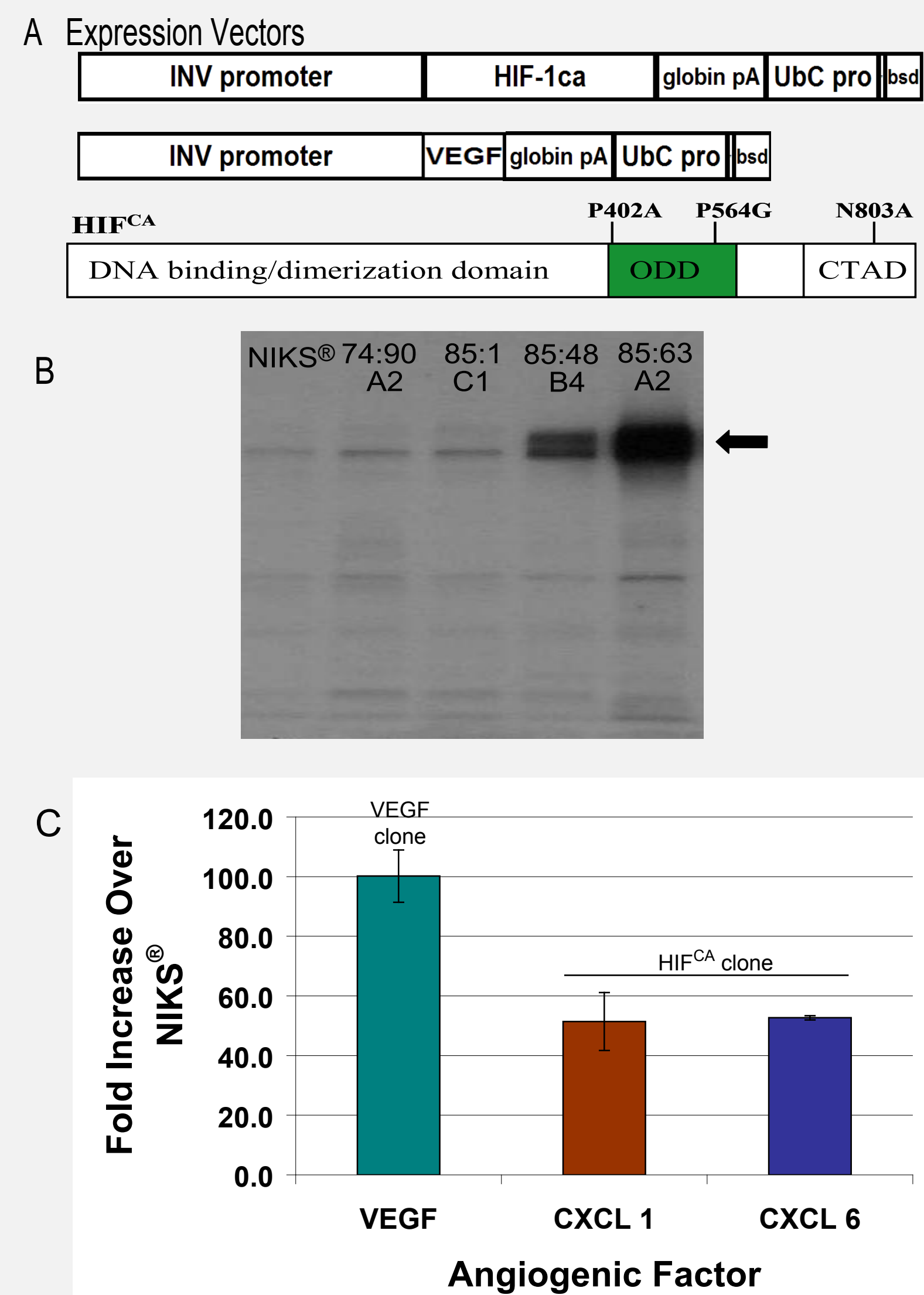
Clonally-pure isolates of stably-transfected cells were identified that each had the expression vector integrated at a single site. Genetically-modified skin substitutes expressed VEGF at levels ~100 fold higher than unmodified tissue and secreted elevated levels of several pro-angiogenic CXCL chemokines (CXCL1 and CXCL6). Conditioned medium from ExpressGraft^{vascular} tissue stimulated the proliferation of human microvascular endothelial cells compared to medium from unmodified skin substitutes. ExpressGraft^{vascular} tissue was vascularized to a greater degree than unmodified skin substitute tissue following engraftment on diabetic and nude mice. Importantly, the increased vasculature showed no evidence of abnormal vessel permeability.

Conclusions

ExpressGraft^{vascular} skin substitute tissue prepared from a consistent, homogeneous keratinocyte source stably modified with a non-viral vector offers significant advantages in product safety and uniformity compared to products prepared from variable keratinocyte populations heterogeneously modified with viral vectors. The ability of skin substitutes expressing elevated levels of angiogenic factors to promote increased vascularization *in vivo* supports the hypothesis that these substitutes may accelerate the vascularization and healing of acute traumatic and chronic wounds.

Results

Figure 1 Stably-Transfected NIKS[®] Cells Over-express Angiogenic Factors

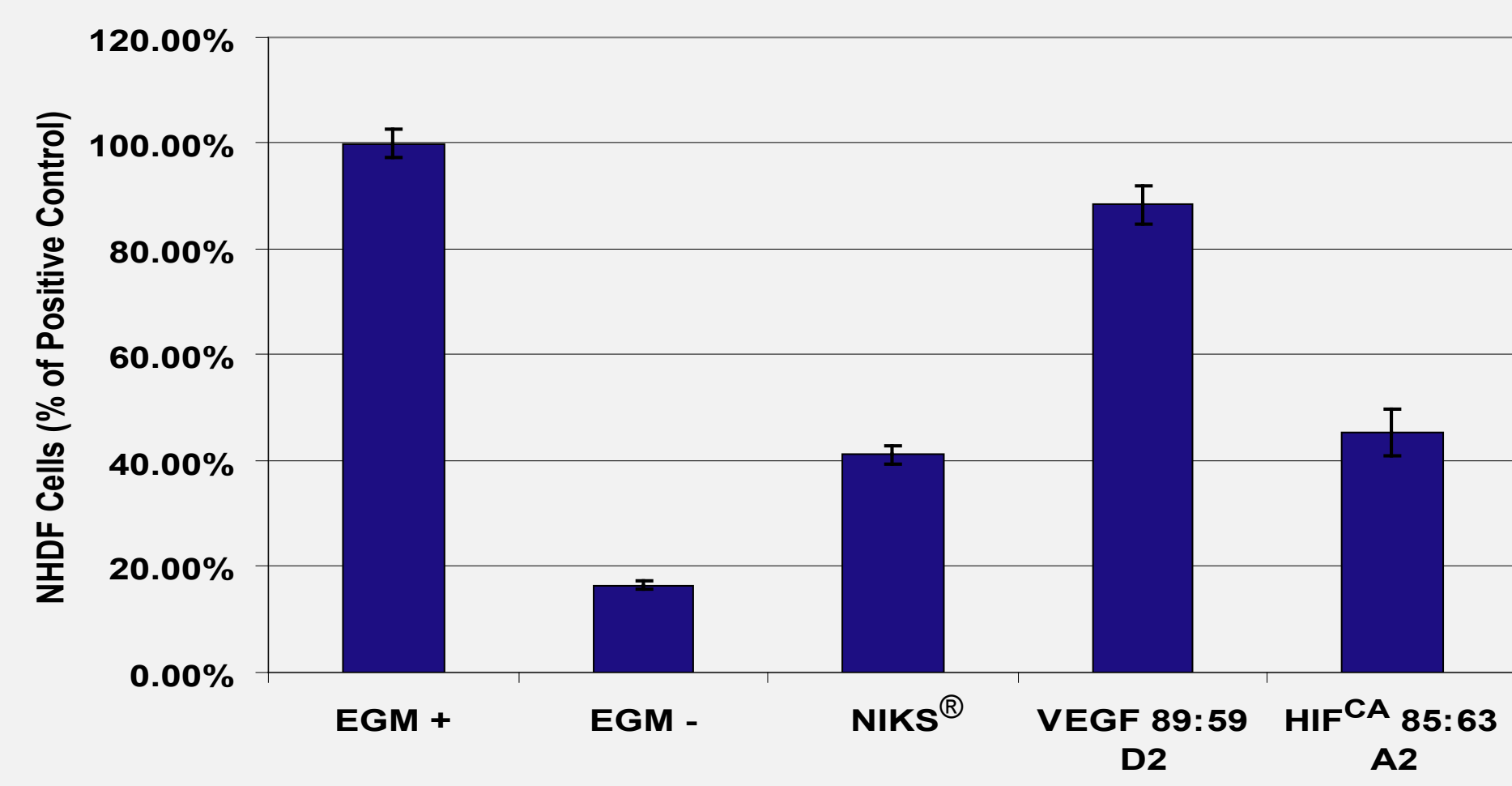


(A) NIKS[®] cells were transfected with non-viral expression vectors encoding a stabilized variant of HIF-1 α (HIF^{CA}) or VEGF₁₆₅ and individual clones of stably-transfected cells were recovered. Point mutations resulting in the constitutively-active HIF^{CA} protein are shown.

(B) Western blot showing the elevated levels of HIF^{CA} (arrow) in different HIF^{CA} clones. Data presented in the remaining figures is for the clone expressing the highest level of HIF^{CA} protein.

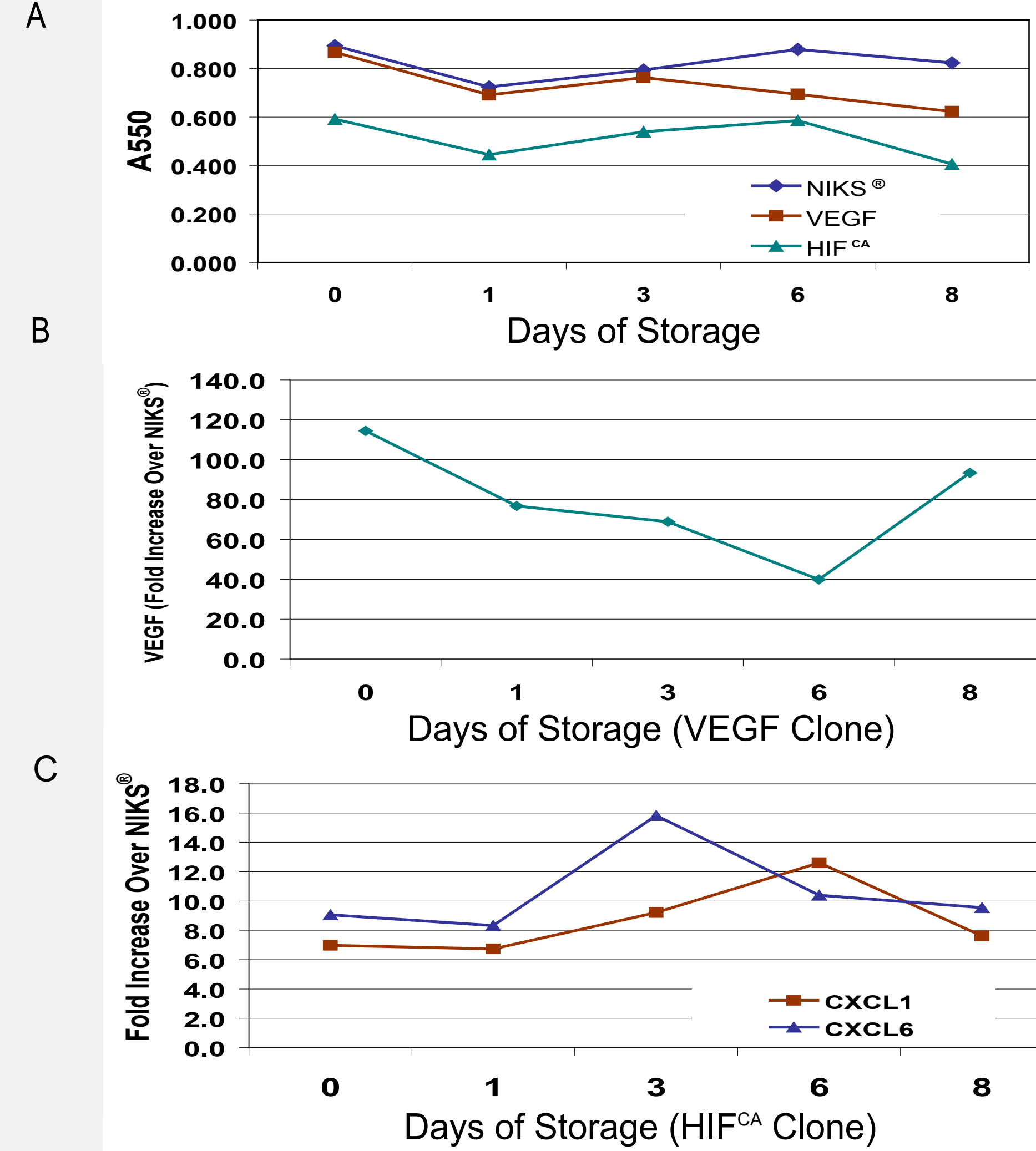
(C) Relative levels of angiogenic cytokines secreted from ExpressGraft^{vascular} tissue normalized to endogenous levels secreted by unmodified NIKS[®] tissue. Cytokine levels were quantified by ELISA. Tissue prepared from cells stably-transfected with the VEGF₁₆₅ vector express elevated levels of VEGF, but no other angiogenic factors. Tissue expressing HIF^{CA} secretes elevated levels of several pro-angiogenic cytokines, but does not express elevated levels of VEGF. Expression data is the average of four independent tissue batches.

Figure 6 Conditioned Medium From ExpressGraft^{vascular} Tissue Stimulates Endothelial Cell Growth



Conditioned medium was collected from unmodified NIKS[®] skin substitutes or tissue made with clones expressing VEGF or HIF^{CA} and tested for the ability to promote the growth of human microvascular endothelial cells (HuMVEC). Positive control medium (EGM+) is fully-supplemented endothelial growth medium, while the negative control (EGM-) is unsupplemented endothelial basal medium. Conditioned medium from tissue was diluted 1:5 with unsupplemented EGM- medium and used to culture HuMVEC cells for 4 days, at which time cells were harvested and counted. Medium from unmodified NIKS[®] tissue stimulates HuMVEC growth compared to EGM- medium due to endogenous production of VEGF and other angiogenic factors. Medium from VEGF-expressing tissue stimulates HuMVEC growth twice as much as unmodified NIKS[®] tissue and five times as much as the negative control (EGM-). This level of stimulation is comparable to that seen with fully-supplemented EGM+ medium. Medium from tissue expressing HIF^{CA} does not stimulate HuMVEC proliferation beyond that seen with unmodified NIKS[®] tissue.

Figure 2 Viability and Angiogenic Factor Expression Are Maintained After Storage of ExpressGraft^{vascular}



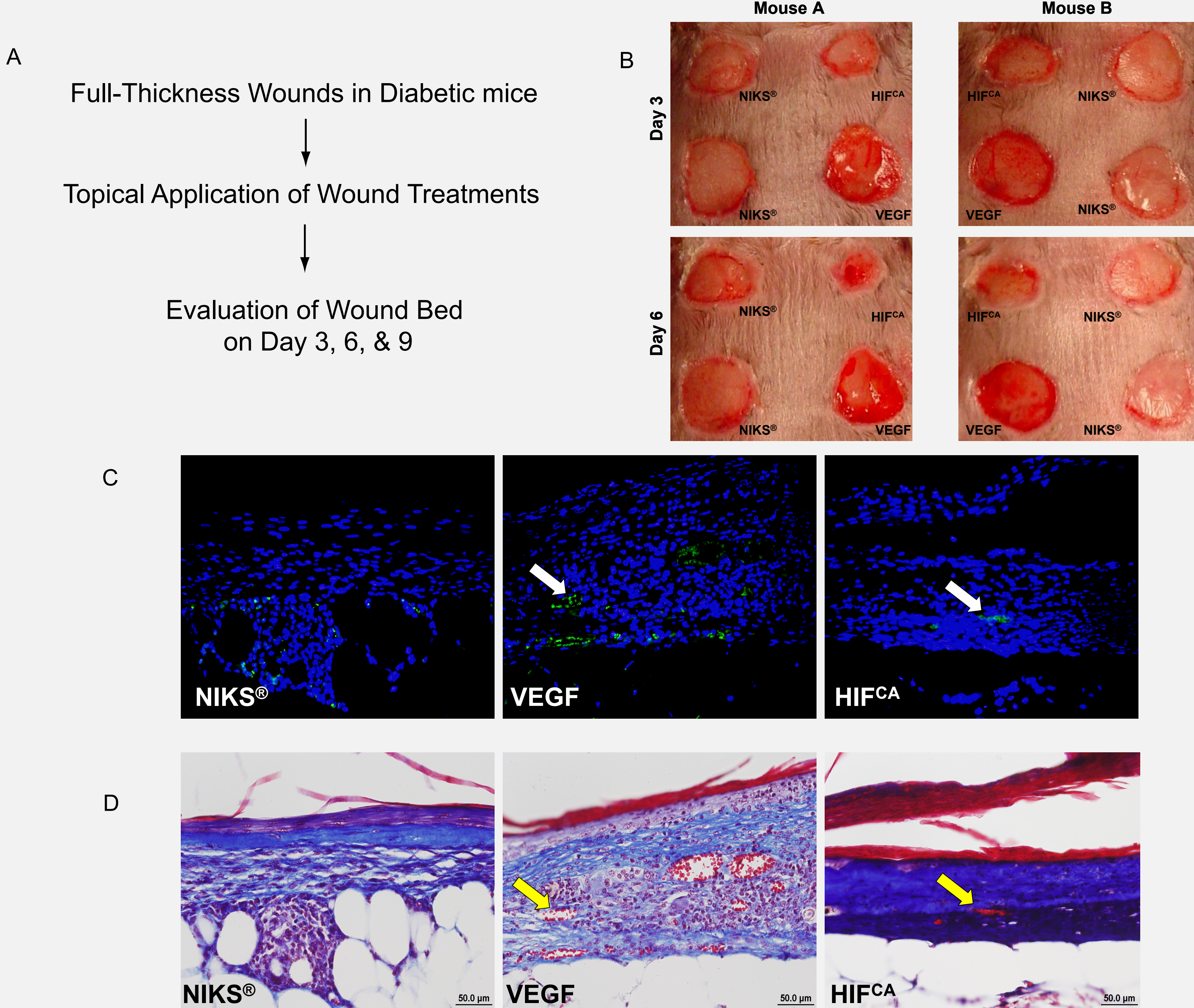
Mature skin substitute tissues were placed in refrigerated storage and samples were obtained after 1, 3, 6, or 8 days and returned to culture for 24 hours.

(A) Tissue viability was measured by the ability of metabolically-active cells within the tissue to reduce MTT. The extent of MTT reduction was quantified by measuring the absorbance of tissue extracts at 550 nm.

(B,C) The levels of angiogenic factors secreted into conditioned medium by ExpressGraft^{vascular} tissue were determined by ELISA and compared to the endogenous levels of these factors secreted by unmodified NIKS[®] tissue. Values are presented as the fold difference between tissue modified to express VEGF (B) or tissue expressing the HIF^{CA} protein (C).

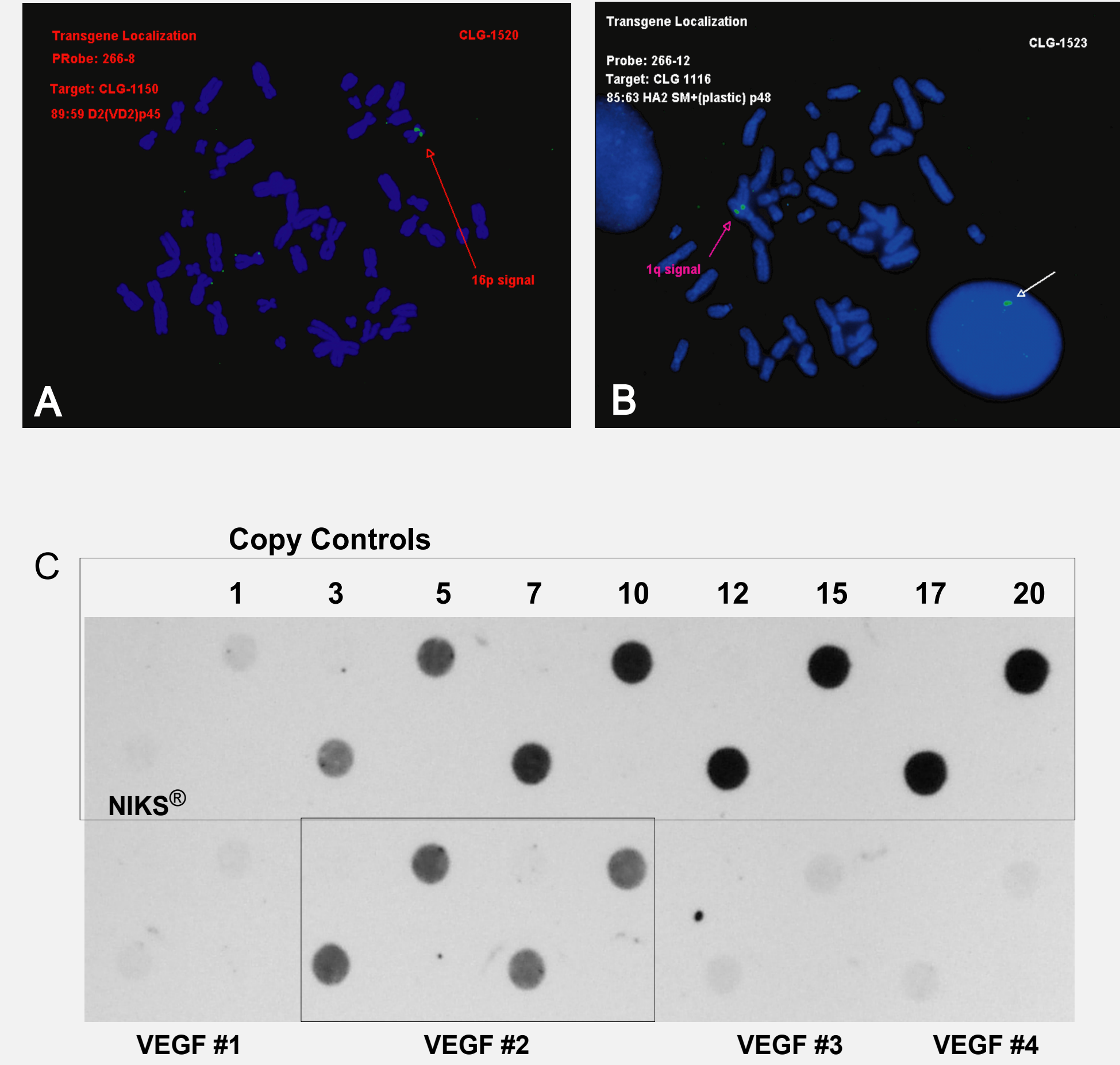
Enhanced cytokine levels are maintained for at least 8 days of storage

Figure 7 ExpressGraft^{vascular} Tissue Promotes Wound Vascularization in Diabetic Mice



Experimental design is shown in (A). Unmodified NIKS[®] tissue or ExpressGraft^{vascular} tissue was applied to full-thickness wounds on diabetic (Lepr^{db}) mice. Three, six, and nine days post-grafting, skin substitutes were removed and the vascular appearance of the wound bed was assessed (Day 9 data not shown). (B) Wounds treated with unmodified NIKS[®] tissues exhibit vascularization only at the wound periphery. Wounds treated with VEGF tissue show vascularization throughout the wound bed. Although not as pronounced, wounds treated with HIF^{CA} tissue exhibit a modest vascularization response, with increased vascularization located primarily at the wound margin. Tissues were sectioned and analyzed by indirect immunofluorescence with anti-CD31/PECAM (C) and for tissue architecture with Masson's Trichrome (D). All wounds, independent of treatment condition, show a development of granular tissue and migration or penetration of endothelial cells into the underlying wound bed and dermal compartment. VEGF tissues exhibit the most robust response of endothelial cells and organization of vessels (arrows). HIF^{CA} treated wounds show a more modest response of vessel organization. The control NIKS[®] tissue elicits a more limited response, with individual endothelial cells migrating into the wound bed, but few distinct vessels migrating toward the tissue.

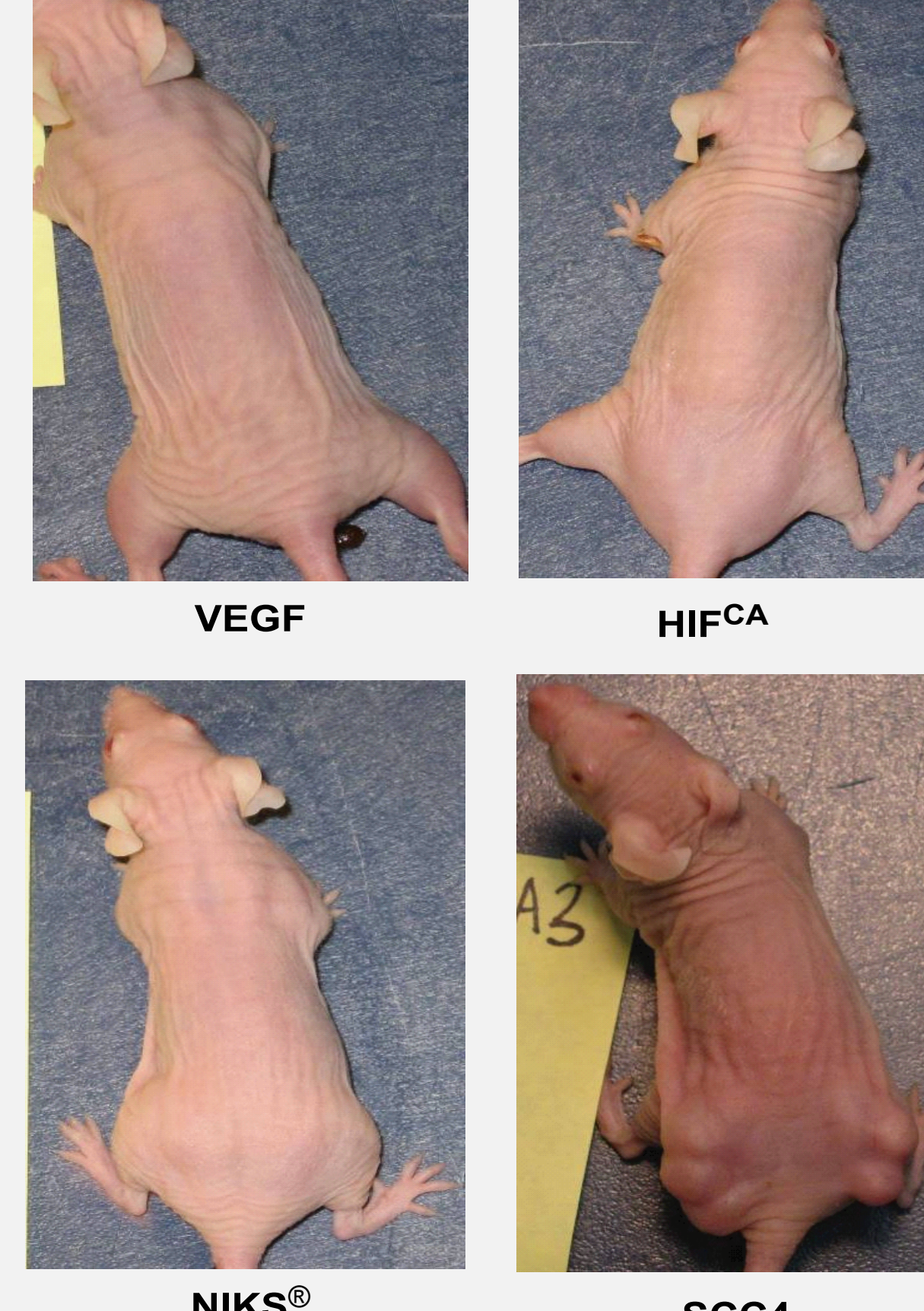
Figure 3 Identification of Transgene Integration Site and Copy Number



(A, B) Transgene integration site was determined by fluorescence in situ hybridization (FISH) using probes prepared from the vectors used to isolate the stably-transfected VEGF or HIF^{CA} clones. The VEGF clone contains a single detectable signal localized to the short arm of one copy of chromosome 16 (A), while the HIF^{CA} (B) clone contains a single detectable signal on the long arm of one copy of chromosome 1. The intensity of the fluorescent signal suggests that the integration sites for both clones most likely contain multiple copies of the corresponding transgene.

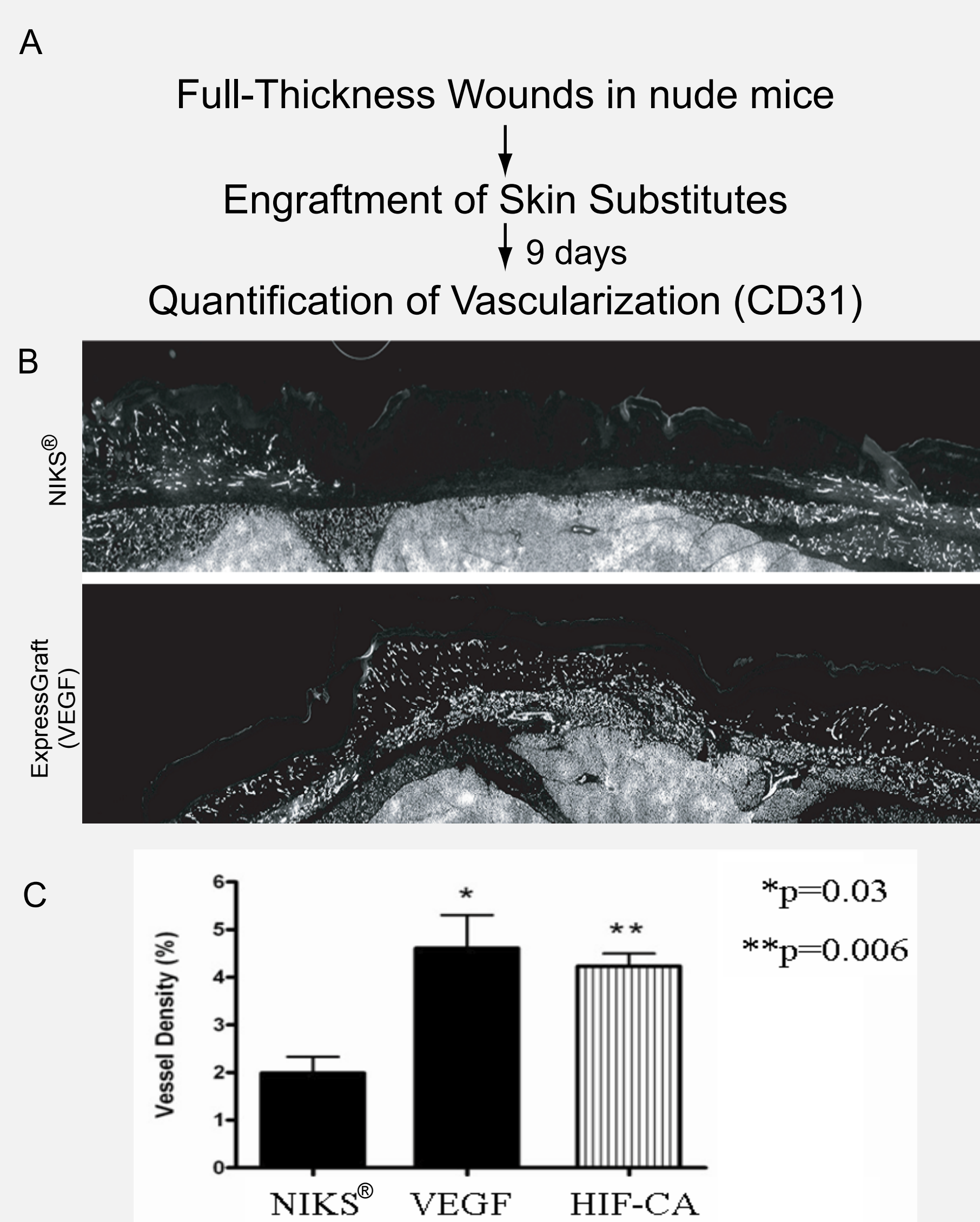
(C) Transgene copy number for several VEGF clones was confirmed by DNA dot blot compared to a transgene-specific copy number dilution series (top two rows). The VEGF clone used for these studies (VEGF #2, in box on bottom rows) has 3-5 copies of the transgene. Transgene copy analysis of the HIF^{CA} clone is in progress.

Figure 4 ExpressGraft^{vascular} Clones Are Non-Tumorigenic



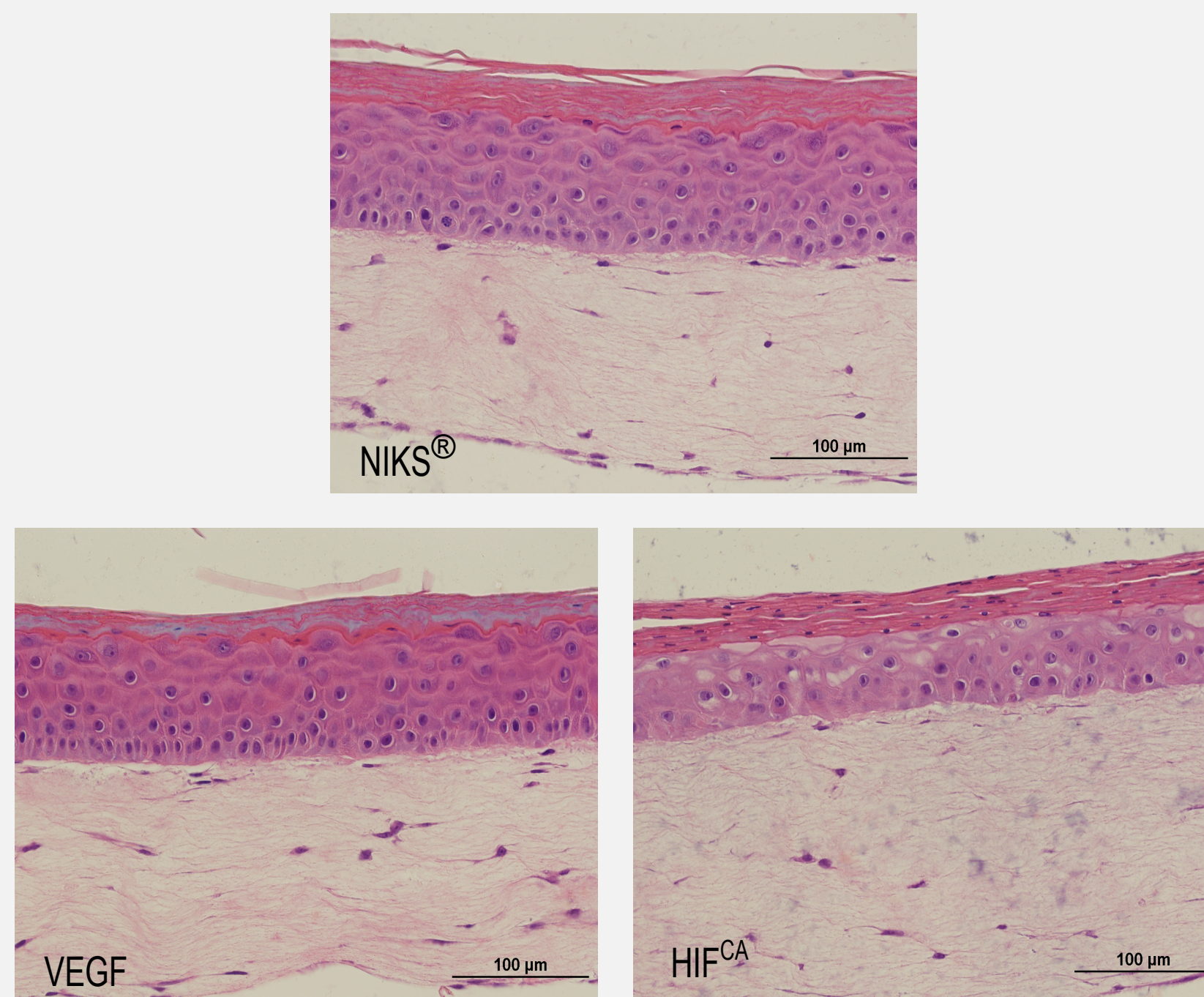
The tumorigenic potential of NIKS[®] cells and stably-transfected VEGF or HIF^{CA} clones was evaluated by injecting NIKS[®], VEGF and HIF^{CA} cells subcutaneously into the flanks of athymic nude mice. No tumors derived from NIKS[®], VEGF or HIF^{CA} cells were found in the study mice, indicating that these cells are not tumorigenic. As a control, cells from the squamous cell carcinoma line SCC4 formed progressively growing tumors when injected into a separate group of control mice. In addition, these clones did not exhibit anchorage-independent growth in soft agar (not shown), confirming the lack of tumor formation in nude mice. Cytogenetic analysis performed on the VEGF and HIF^{CA} clones at various passages has demonstrated that these clones maintain the same stable karyotype that is characteristic of the parental NIKS[®] cells.

Figure 8 Quantitative Analysis of Enhanced Vascularization of ExpressGraft^{vascular} Tissue *in vivo*



Experimental design is shown in (A). Skin substitutes prepared from unmodified NIKS[®] cells or clones expressing VEGF or HIF^{CA} were grafted onto full-thickness wounds in athymic nude mice. After nine days, grafted tissue was harvested and vascular density was quantified by immunofluorescence detection of CD31. Representative photographs of CD31 staining are shown in (B). Vessel density in the central region of the grafted tissue is greater in ExpressGraft^{vascular} tissue compared to tissue prepared from unmodified NIKS[®] cells. Quantification of the CD31 staining (C) shows that ExpressGraft^{vascular} tissue prepared from both the VEGF and HIF^{CA} clones results in statistically-significant increases in vascular density compared to unmodified NIKS[®] tissue. Fluorescently staining vessels were quantified using image intensity threshold analysis, with statistical analysis determined by one-way ANOVA and the Bonferroni test for multiple comparisons.

Figure 5 Epidermal Differentiation of ExpressGraft^{vascular} Tissue



Sections of tissue produced by unmodified NIKS[®] cells or clones expressing VEGF or HIF^{CA} were stained with hematoxylin and eosin to visualize epidermal architecture. Tissue prepared from NIKS[®] cells and the VEGF clone exhibit normal epidermal differentiation, resulting in a stratified epidermal layer with well-defined basal, spinous, granular, and cornified layers. Tissue prepared from the HIF^{CA} clone exhibits altered (parakeratotic) epidermal differentiation, with a thinner epidermal layer and nucleated cells in the stratum corneum.

Summary

Stably-transfected clones of the clinically-tested NIKS[®] epidermal progenitor cell line were isolated with non-viral vectors encoding VEGF or a constitutively-active variant of HIF-1 α .

Skin substitutes generated with stably-transfected NIKS[®] cells secrete multiple angiogenic factors at levels ~50-100 fold higher than unmodified skin substitutes

Skin substitutes generated with stably-transfected NIKS[®] cells stimulate endothelial cell proliferation *in vitro* and promote wound vascularization *in vivo*.

Conclusions

- The long-lived NIKS[®] epidermal progenitor cell line is an ideal source of cells for tissue engineering and is amenable to modification with non-viral vectors
- Second-generation skin substitutes prepared from genetically-modified NIKS[®] cells are promising candidates to enhance the vascularization and healing of acute and chronic wounds

Acknowledgements

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